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Introduction

Flavopiridol (FP) is an investigational new drug currently in Phase II clinical trials for the treatment of solid tumors (Senderowicz *et al.*, 1998). The precise mechanism of action of this compound is unknown, however, it is known to inhibit several members of the cyclin-dependent protein kinase family, and to induce cell death (Senderowicz, 1999, Schrump, *et al.*, 1998, Bible and Kaufmann, 1996). The objective of this proposal is to gain insight into the molecular mechanism(s) whereby human tumor cells become resistant to FP. To gain greater insight into this question, a FP-resistant clone was generated from the human MCF-7 breast adenocarcinoma cell line. This clone was obtained by exposing growing cultures of MCF-7 cells to increasing concentrations of FP over a several month period of time. The resulting clone, named MCF-7/F, has an IC_{50} for FP that is 24-fold lower than that of the parental MCF-7 cell line cell line from which it was derived. Specifically, the experiments described in this proposal are designed to identify the molecular basis for FP resistance in MCF-7/FP cells. We have evaluated, or are in the process of evaluating cellular levels of known FP targets, i.e. cyclin-dependent protein kinases in MCF-7/FP and MCF-7 cells. In addition, we have begun to examine the relative resistance of these cells to a number of drugs. Finally, we have been examining the relative expression levels of a number of drug efflux pumps in these cell lines. It is anticipated that FP or FP-like molecules will ultimately assume a place in the modern cancer chemotherapeutic armamentarium. Thus, insight gained into the molecular basis of FP drug resistance in MCF-7/FP cells may ultimately prove beneficial in the design of second or third generation FP analogues. It is also conceivable that this information may aid in the development of chemotherapy strategies to minimize the emergence of clinical resistance to these agents.

Body

Task 1. Ascertain whether human normal breast epithelial MCF-10A cells are, relative to human breast adenocarcinoma MCF-7 cells, less sensitive to FP.

> Preliminary results relayed to me by Dr. Normal Sladek (personal communication), the original PI of this proposal indicate that, contrary to what was anticipated, the MCF-10A cells line is actually several fold more sensitive to the cytotoxic effects of FP than are MCF-7/0 cells. Our preliminary results (data not shown) appeared to confirm this finding. Since we are making substantial progress in a number of other areas (see below), we are not currently pursuing this objective. This approach may be reconsidered in the future, based upon the outcome of the experiments described below.

Task 2. Ascertain whether stable resistance to FP on the part of MCF-7/FP cells persists beyond 90 cell divisions.

We grew MCF-7/FP cells for 20 additional cell divisions (beyond the original 90) in the absence of FP. Cytotoxicity assays revealed no significant loss of FP resistance compared to cells that had either not been grown, or had been grown in the presence of FP (data not shown). Based on these results we have tentatively concluded that the FP resistance observed in MCF-7/FP cells should be termed 'stable'. It does not seem fruitful to pursue this approach any further at this time.

Task 3. Ascertain whether the sensitive MCF-7 cell line and the insensitive MCF-7/FP subline differ in selected cell cycle parameters.

Two distinct series of experiments have been performed. First, the cellular doubling time of the MCF-7 and MCF-7/FP cell lines were determined. As Figure 1 indicates, there is no apparent difference between the two cell lines with respect to this parameter. (FIGURES AND FIGURE LEGENDS ARE LOCATED IN THE APPENDIX) We have also been performing FACS analyses of these cells to determine the relative proportions of the cells that are in the G1 and G2 portions of the cell cycle. While these results are preliminary, there appears to be some subtle differences in the distribution within the phases of the cell cycle between the two cell lines (not shown). We are continuing to examine this issue, and expect to have firm results within the next 1-2 months. In addition, we intend to examine whether exposure to equi-toxic concentrations of FP differentially perturbs these cell cycle parameters in the two cell lines.

Task 4. Ascertain whether MCF7/FP cells are cross-resistant to flavopiridol analogues, other flavone anticancer agents, UCN-01, and/or anticancer agents presently used to treat metastatic breast cancer.

We have initiated a series of experiments to address this objective. The resistance of the MCF-7/FP and MCF-7 cells to cisplatin was examined. As Figure 2 (APPENDIX) indicates the FP-resistant cells show no cross-resistance to cisplatin. (We have also performed analogous experiments examining resistance to mitoxanthrone. These results indicate that the MCF-7/FP cells are cross-resistant to this drug. However, we have not yet performed sufficient numbers of experiments to quantitate the level of resistance, thus these data are not presented herein.) We intend to perform additional experiments of this type, determining

the resistance of these two cell lines to compounds including FP analogs, UCN-01 and other flavone anticancer agents. I anticipate that these experiments will require an additional 6 months to complete.

Task 5. Ascertain whether elevated levels of the kinase(s) inhibited by FP account for MCF-7/FP insensitivity to this agent.

We performed western blot analyses using antibodies specific for several of the cyclin-dependent protein kinases. These results are summarized in Figure 3A and 3B (APPENDIX). Figure 3a depicts the results from one representative experiment, while Figure 3b presents more quantitative examination of the results from several independent experiments. This analysis reveals that the MCF-7/FP cell line does not have elevated levels of cdks 2, 4 or 7, compared to the MCF-7 parental cell line. In contrast, we observed a modest, but consistent increase in the level of cdc2 (cdk1) protein level in the MCF-7/FP cells. The differences in cdc2 protein levels seen in the two cell lines do not rise to the level of statistical significance (P=0.12, 1-tailed paired T test). However, in our experience, this type of analysis is associated with large standard errors. Therefore, we are in the process of repeating this analysis on several more independently prepared samples. We hypothesize that this increased analysis will support the tentative conclusion that cdc2 levels are modestly elevated in the MCF-7/FP cell line. (In addition, we intend to perform northern blot analysis using a cdc2 cDNA probe to further address this question.)

In the coming months we will be examining cellular protein levels of other cdks, such as cdk8 and cdk9. In addition, we have been examining the relative levels of a number of cyclins including cyclin D1, and cyclin D3. Our tentative conclusion from these experiments is that levels of these cyclins are not elevated in the MCF-7/FP cell line, relative to the parental MCF-7 cells. We've not yet performed these experiments a sufficient number of times, however, to convincingly support this conclusion. We anticipate that this analysis will be completed within the next month or so.

Task 6 Ascertain whether an elevated level of a drug transport pump, a drug metabolizing enzyme and/or a nontarget flavopiridol-binding protein accounts for MCF-7/FP insensitivity to flavopiridol.

We have used northern blot analysis to examine the level of the ABCG2 transporter in MCF-7/FP and MCF-7 cells. This analysis reveals that the former

have approximately 3-fold greater levels of Abcg2 mRNA than do the later (Figure 4, APPENDIX). Consistent with this finding, western blot analysis revealed an approximately similar level of increase in Abcg2 protein levels in the resistant cell line (not shown).

Using a qualitative drug efflux assay, we examined the relative efficiency with which the MCF-7/FP and MCF-7 cell lines were able to remove the fluorescent calcein analog calceinAM and rhodamine123. Representative results presented in Figure 5 (APPENDIX) revealed no difference in the efflux of rhodamine 123 from MCF-7/FP or MCF-7 cells. In contrast, MCF-7/FP cells were far more efficient in calceinAM efflux than were MCF-7 cells (Figure 5, APPENDIX). These results are only suggestive, and we are in the process of performing more quantitative tests to confirm these findings. However, if these results are confirmed, they would suggest that the MCF-7/FP cells do not have elevated levels of the multidrug resistance1 protein (Mdr1). They also suggest that these cells may have elevated levels of the one of the multidrug resistance-associated proteins (Mprs). Interestingly, Robey *et al.* (2000) showed that a cell line overexpressing the MPR1 gene was not resistant to FP, suggesting that the calceinAM efflux activity seen in the MCF-7/FP cells is not likely to be due to overexpression of this gene. Instead, it appears likely that overexpression of another drug efflux pump, perhaps another member of the MPR family, may be responsible for the calceinAM efflux phenotype seen in the MCF-7/FP cell line. We intend to perform northern blot analysis on mRNA from these cells using MDR and MPR cDNA probes to address this question. It is anticipated that this analysis will occur over the next 6-12 months.

Task 7. As was outlined in the original proposal, this tasks will only be pursued if completion of Tasks 5 and 6 do not provide a full phenotypic explanation for the acquired resistance of the MCF-7/FP cells. These studies are still underway, thus, we do not anticipate addressing this objective for several months, at least.

Key Research Accomplishments. Results obtained during the most current year of support permit us to reach the following conclusions.

1. The drug resistance seen in the MCF-7/FP cells appears to be stably expressed even following prolonged cell doubling in the absence of selection.
2. The cell doubling time of the MCF-7/FP cells does not differ significantly from that of the parental MCF-7 cells. However, the distribution of the two cell lines within the distinct phases of the cell cycle does appear to differ.

3. MCF-7/FP cells are resistant to mitoxanthrone. In contrast, these cells are no more resistant to the cytotoxic effects of cisplatin than are the control MCF-7 cells.
4. MCF-7/FP cells appear to have a small increase in the level of cdc2 protein, compared to MCF-7 cells. Levels of other cdks appear unaltered.
5. MCF-7/FP cells have an approximate three-fold increase in the level of ABCG2 mRNA, compared to MCF-7 cells.
6. Preliminary results suggest that FP resistance in the MCF-7/FP cells may be associated with overexpression of an MPR-like gene (although MRP-1 is probably not elevated). In contrast, the MCF-7/FP cells do not appear to have significantly enhanced MDR1 gene expression, compared to the MCF-7 cells.

Reportable Outcomes. None

Conclusions. There appear to be numerous mechanisms through which cells can become resistant to the cytotoxic effects of FP. It has been well established that overexpression of the ABCG gene may lead to resistance (Robey *et al.*, 2001). However, the drug resistance profile of other FP-resistant cell lines suggests that their resistance to FP may not be due to a similar mechanism, since they failed to detect any evidence for reduced FP accumulation in this cell line (Smith *et al.*, 2001). In addition, Bible *et al.* (2000) described an ovarian cell line that appears to be resistant to FP through yet another mechanism. In contrast to the cell lines described by Robey *et al.* and Smith *et al.*, (and that which we have described) the ovarian cancer cell line acquired FP resistance spontaneously. In addition to being FP resistant, these ovarian cells were also resistant to cisplatin, a phenotype not associated with the other FP-resistant cell lines. Furthermore, these cells were not resistant to mitoxanthrone. Thus, while direct evidence is lacking, these findings suggest that the mechanism through which these ovarian cancer cells became resistant to FP, although apparently due to reduced intracellular accumulation of drug, is nevertheless unlikely to have resulted from overexpression of the ABCG2 gene.

The phenotype of the MCF-7/FP cells suggests that the mechanism through which they have become FP resistant is at least partially distinct from that associated with the FP-resistant cell lines discussed above. While overexpression of the ABCG2 transporter is seen in the MCF-7/FP cells, the level of overexpression is far lower than that described by Robey *et al.* This finding is particularly intriguing since the Robey group used an essentially identical protocol to generate the drug-resistant MCF-7 derivative cell line (which they called MCF-7 FLV1000). Robey *et al.* used RT-PCR to determine that the MCF-7 FLV1000 cells had a nearly 50-fold increase in ABCG2 mRNA levels, compared to the parental MCF-7 cell line. (Inspection of the northern blot data presented in their paper suggests this may actually be a minimal estimate of the degree of overexpression of

this gene.) In contrast, the northern blot analysis of RNA from the MCF-7/FP and MCF-7 cells described herein indicates that levels of ABCG2 gene expression in the former are elevated no more that 3-fold over those seen in the latter. Additional findings support the idea that ABCG2 gene expression levels in MCF-7/FP cells are not elevated to the same extent as that seen in the MCF-7 FLV1000 cells described by Robey *et al.* Whereas the FLV1000 cells were nearly 600-fold more resistant to mitoxanthrone than were MCF-7 cells, the MCF-7/FP cells do not appear to display this level of resistance to this compound, compared to MCF-7 cells.

These preceding discussion highlights that resistance to FP can occur *via* multiple mechanisms. Perhaps more provocatively, these results suggest that multiple mechanisms of FP resistance may function within a single cell. For example, the data included in this report are consistent with the conclusion that while overexpression of the ABCG2 drug transporter contributes to the FP resistance seen in MCF-7/FP cells, there are likely to be additional drug resistance mechanisms at work in these cells. This finding is of great significance. It implies that not only may that clinical resistance to FP arise via multiple mechanisms amongst different patients, but that within a single patient there may be multiply resistant cancer cells. If FP or FP analogs are to eventually become important anticancer agents, it is imperative that the mechanisms through which these distinct resistance pathways function be fully understood.

The availability of the MCF-7/FP cell line will permit us to address this issue in greater detail. The approaches outlined above will be pursued over the next period of support. In addition, we are in the process of generating additional FP resistant clones. The protocol used to isolate MCF-7/FP cell line (in which the cells were serially transferred through increasing concentrations of FP over a prolonged period of time) may have contributed to the evolution of multiple mechanisms of resistance. Therefore we are utilizing two different protocols to generate additional cell lines. First, we are trying to obtain clones resistant to intermediate levels of FP via a one-step procedure. It is hoped that this approach will allow us to isolate clones that have only one single mechanism of drug resistance. In this approach we are encouraged by the finding that within a single genetic background (the MCF-7 cell line) two different groups were able to obtain cells with apparently distinct FP resistance profiles.

The second strategy we will utilize is identical to that used to originally obtain the MCF-7/FP cell line. However, in this case, we will be isolating clones throughout the dose-escalation process. Again, it is our hope that this strategy will permit us to isolate clones that, while perhaps relatively less resistant to FP than the MCF-7/FP clone will prove to be resistant due to a single (possibly novel) resistance mechanism.

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APPENDIX.
FIGURE LEGENDS

Figure 1. *MCF-7 and MCF-7/FP cells have similar doubling times.* 100,000 cells were plated in triplicate onto 10 cm dishes in rich media. Cells were harvested by treatment with trypsin and counted (as replicates of three) on successive days during which time log growth was observed. The slope of the growth curve was used to calculate the doubling time. The data presented represent the mean ± SEM from three separate growth experiments.

Figure 2. *MCF-7/FP cells are not cross-resistant to cisplatin.* 3000 cells were plated on 10 cm dishes (in triplicate). 24 hours later, the cells were exposed to the indicated concentrations of cisplatin. The following day, the drug was washed out, and the cells allowed to form colonies. 15 days later, colonies were counted by staining with crystal violet. The results represent the averages obtained from three separate experiments.

Figure 3. *Analysis of cyclin-dependent protein kinases in MCF-7/FP and MCF-7 cells.* 10 micrograms of whole cell lysates from the two cell lines were resolved on denaturing SDS polyacrylamide gels, and transferred to nitrocellulose. Incubations were carried out using rabbit polyclonal antibodies (at 1:1000 dilutions) specific for the proteins indicated. Incubation with an alkaline phosphatase-conjugated goat-anti rabbit second antibody was performed, and the BCIP reaction utilized for colorimetric determination. The image labeled (A) depicts representative data from a single western blot analysis. Image (B) depicts the results obtained from scanning densitometric examination of data from three sets of western blot experiments. Error bars represent the SEM.

Figure 4. *MCF-7/FP cells overexpress the ABCG2 gene.* Northern blot analysis was performed on 20 micrograms of cytoplasmic RNA isolated from MCF-7 and MCF-7/FP cells. Duplicate samples were hybridized with probes specific for GAPDH and ABCG2. This analysis was performed three times using different RNA preparations. Scanning densitometry was performed using a phosphoimager and IP labgel software. Levels of hybridization by the two probes observed with the MCF-7/FP cell samples were normalized to those observed with the MCF-7 cells. Error bars depict the SEM.

Figure 5. *Efflux of CalceinAM is enhanced in MCF-7/FP cells.* MCF-7/FP and MCF-7 cells were incubated in the presence of 1micromolar solutions of either Rhodamine123 or CalceinAM for 60 minutes. Drugs were washed out, and the cells immediately examined using confocal fluorescence microscopy.
MCF-7/FP cells

FIGURE 1

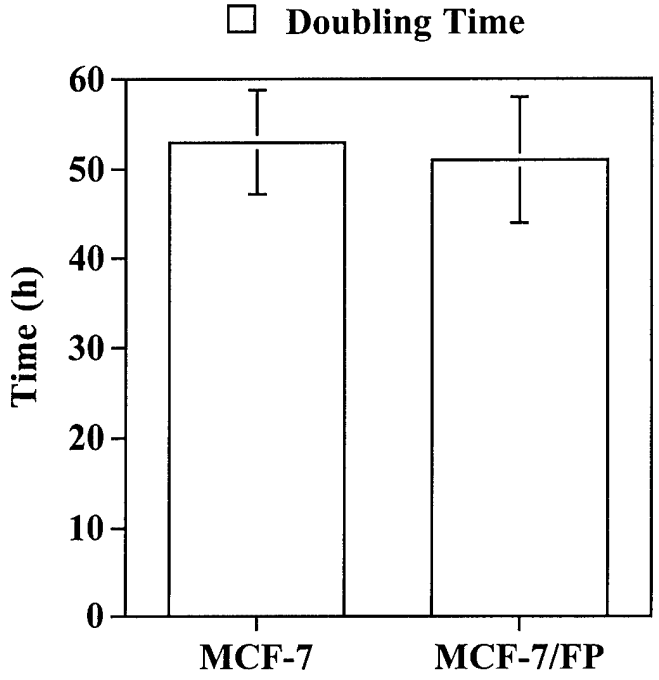


FIGURE 2

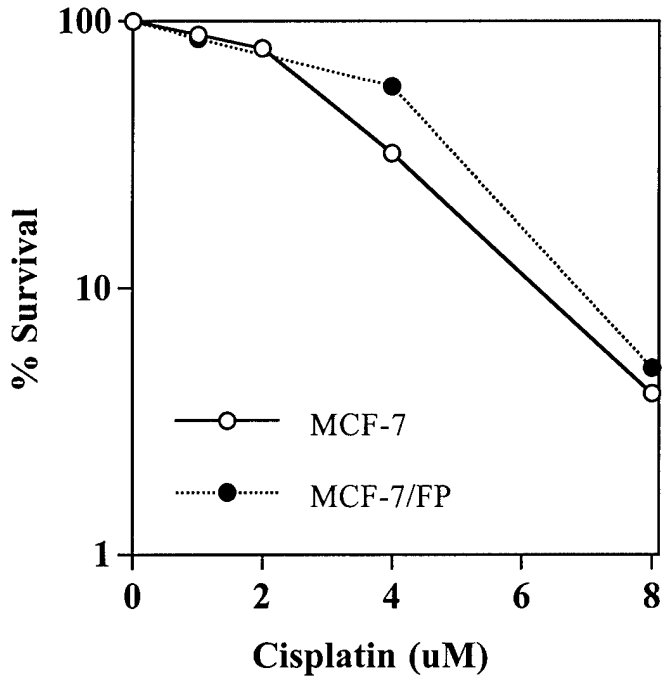


FIGURE 3A

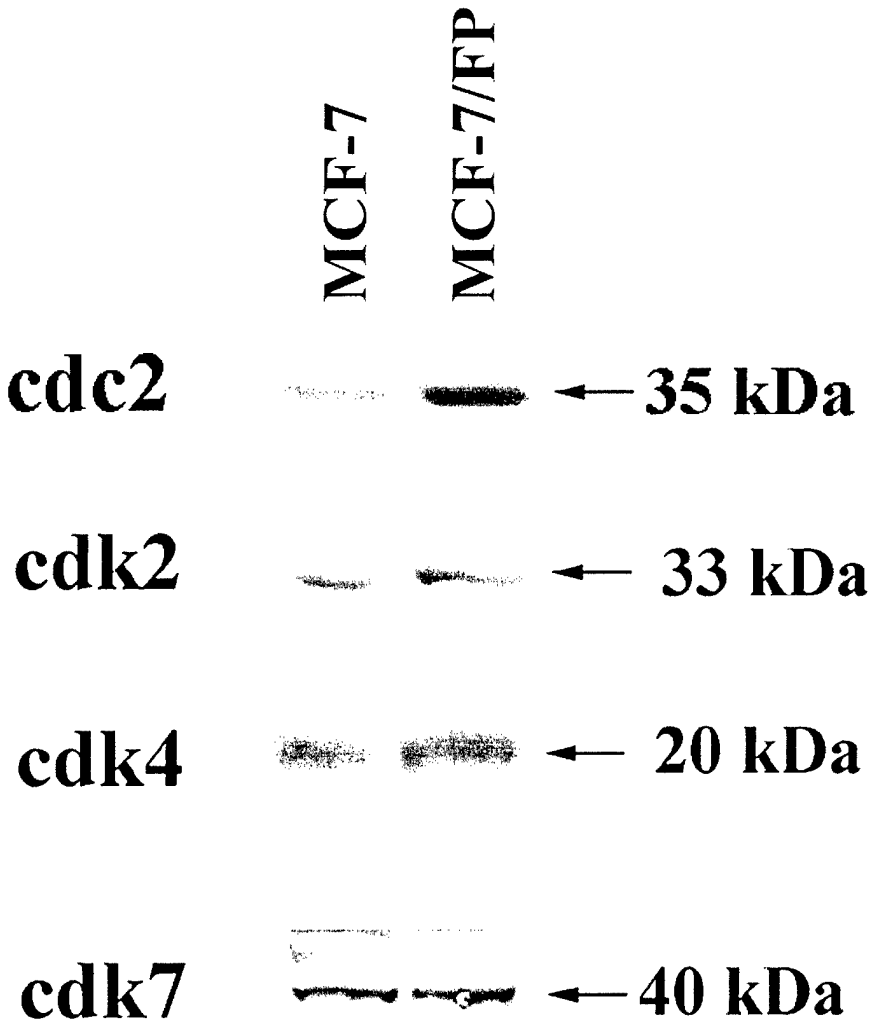
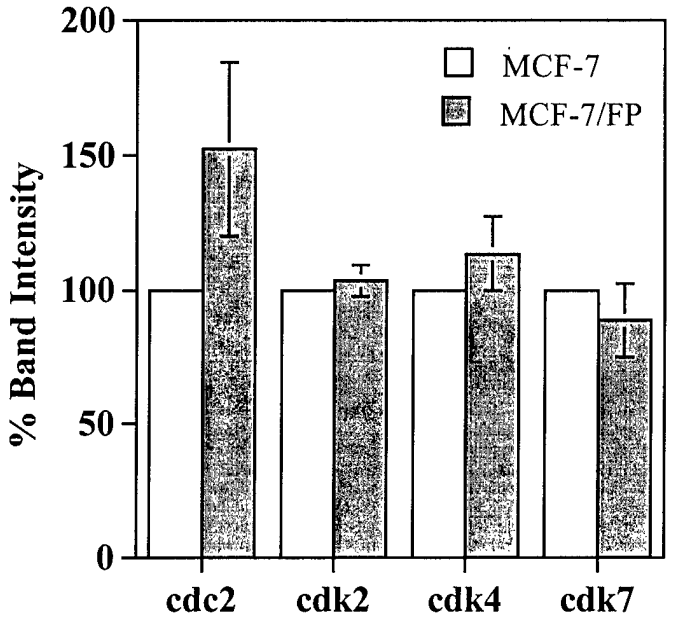


FIGURE 3B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% Band Intensity
MCF-7
MCF-7/FP
cdc2
cdk2
cdk4
cdk7

FIGURE 4

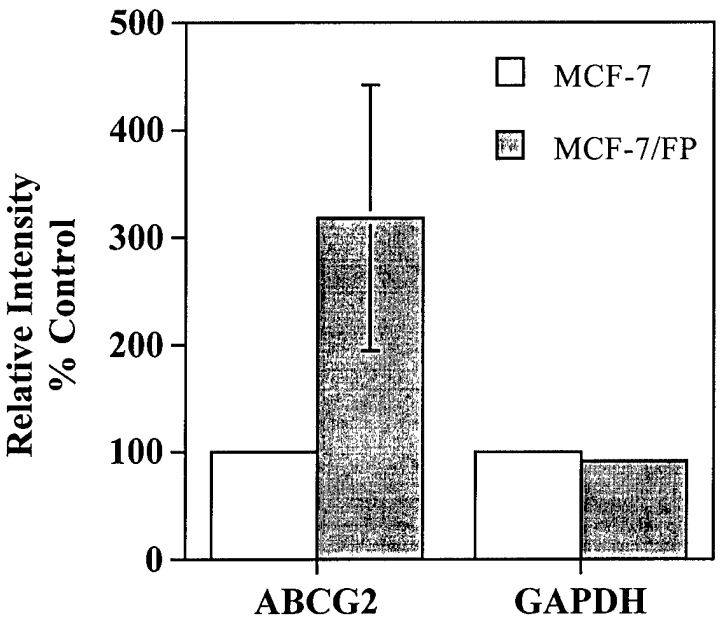
500
400
300
200
100
0
Relative Intensity
% Control
MCF-7
MCF-7/FP
ABCG2
GAPDH

FIGURE 5